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14. ABSTRACT

The objective of the project is to determine the role of endogenous lysophosphatidic acid (LPA) in lipogenesis and metabolic abnormalities of ovarian cancer cells. During the second year of the funding support, the most significant advance we made was the identification of a previously unrecognized functional aspect of LPA-producing enzymes or LPA-synthesizing pathways such as iPLA2 and PLA2-autotaxin. They not only produce bioactive LPA but also regulate the availability of fatty acids for b-oxidation. We have obtained strong evidence that fatty acids and boxidation metabolism play an active role in driving cell proliferation and resistance to apoptosis. One of the key mediators of fatty acid b-oxidation is carnitine pamitoyl transferase 1A (CPT1A), which is overexpressed in malignant ovarian epithelial cells. Our results together indicate a dual role for lipid metabolism (LPA-driving lipogenesis and fatty acid catabolism) in support of malignant features of ovarian cancer cells. We have also obtained interesting results from other aspects of the project as detailed in the progress report. With the 6-month no-extension that we have requested, we will be able to accomplish most of the project objectives.

15. SUBJECT TERMS
LPA, ovarian cancer, lipid metabolism, iPLA2, fatty acids

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Annual Progress Report for W81XWH-11-1-0541, Year 2

Introduction

Increased *de novo* lipogenesis is a metabolic characteristic of ovarian cancer and other human malignancies. The current ovarian cancer pilot project titled “Pro-lipogenic action of lysophosphatidic acid in ovarian cancer” is to determine the role of endogenous lysophosphatidic acid (LPA) in lipogenesis and lipid metabolic abnormalities of ovarian cancer cells. The scopes of research are Task 1: To define the role of endogenous LPA in regulation of lipogenesis in ovarian cancer cells; and Task 2: To determine the contribution of LPA-driven lipogenesis to metabolic abnormalities of ovarian cancer cells. This is a 2-year project ending on 06/30/2013. We have accomplished most of the project objectives. The major and most interesting findings made in the 2nd year are presented or summarized in this report. Only a small portion of the planned studies remains underway due to a change in lab personnel. A manuscript covering the results of the project is currently in preparation. We have therefore submitted to DoD for a no cost extension to the end of this year in order to accomplish all project objectives and to publish our experimental results.

Body of Research Report

Specific Aims:

Aim 1. To define the role of endogenous LPA in regulation of lipogenesis in ovarian cancer cells

1.1 Examination of whether LPA in serum and LPA-inducing agents are sufficient to induce lipogenesis in ovarian cancer cells

In the previous report, we presented evidence that LPA stimulated endogenous lipogenesis via its receptor subtype 2 (LPA2). LPA2 is linked to Gq and G12/13 to activate the AMPK-ACC and SREBP-FAS pathway, respectively as we reported recently (1). The selective role of LPA2 in LPA-mediated activation of lipogenesis enabled us to determine whether other biological fluids such as serum at physiological concentrations could support lipogenesis via its constituent LPA. Indeed, serum stimulated lipogenesis in an LPA2-dependent manner in other ovarian cancer cell lines we tested (data not shown). Certain growth factors and agonists have been reported to induce production of LPA, which could drive lipogenesis via an autocrine mechanism. Thus we examined the effect of EGF on *de novo* lipid synthesis in LPA2-expressing and LPA2-depleted (shRNA) cells. The results indicate that EGF itself could trigger lipogenesis in both experimental conditions with moderate reduction in the absence of LPA2. The data suggest that the autocrine LPA in EGF-conditioned medium might not be sufficient to reach the level to activate lipogenesis via physiologically expressing LPA2 receptor while EGF itself is also a lipogenic factor. The further experiments will be to heat-inactivate the EGF conditioned medium and to use LPA-overexpressing clones of Caov-3, OVCA-432 and SKOV-3 cells to sensitize the cells to low levels of LPA.

1.2 Assessment of the effects of manipulating LPA-producing enzyme autotaxin on activation of lipogenic pathways and *de novo* lipid synthesis in ovarian cancer cells.

We have generated both autotaxin-overexpressing clones and autotaxin-recombinant proteins (2). Autotaxin is one of the primary LPA-generating enzymes (3). Results from these approaches demonstrate that the presence of autotaxin promoted cellular lipogenesis. This part of the work has been completed. The data along with those from other tasks of the current project will be prepared for publication and for an NIH grant application.

1.3 Assessment of the effects on lipogenic enzymes and lipogenesis of pharmacological and molecular inhibition of iPLA2, another enzyme involved in LPA production in ovarian cancer cells.

Because several groups have already reported recently that PLA2 enzymes mediate LPA production and cell growth in various cancer cells (4-6), we focused on the role of iPLA2b-mediated fatty acid production in lipid metabolism and ovarian oncogenesis. The results of this line are summarized together with Task 2.2 which addresses the

significance of the supply of fatty acids for b-oxidation in support of bioenergy and cell cycle progression (see Aim 2.2).

Aim 2. To determine the contribution of LPA-driven lipogenesis to metabolic abnormalities of ovarian cancer cells:

2.1 Analysis of the effects of LPA and LPA production on mitochondrial respiration in ovarian cancer cells

Using an HPLC-based assay, we found that treatment of ovarian cancer cell line with LPA increased ATP production and decreased AMP/ATP ratio, a master regulator of the AMPK activity and energy metabolism in mammalian cells. This part of the study has been completed.

2.2 Elucidation of the role of LPA and LPA production in regulation of lipid catabolic enzymes including monoacylglycerol lipase (MAGL) and fatty acid beta oxidation

The most significant advances have been made in relation to this subaim. We have previously focused on LPA action and production from major LPA-producing enzymes. However, these enzymes and enzymatic pathways such as iPLA2 and PLA2-autotaxin could also lead to release and accumulation of fatty acids, byproduct associated with LPA biosynthesis. We described in the last report that extracellular fatty acids enhances proliferative responses of ovarian cancer cells to growth factors, suggesting that fatty acid availability promotes b-oxidation, energy metabolism and cell proliferation. This hypothetic role of fatty acids released from phospholipases is consistent with our previous observation that exogenously supplemented LPA did not fully reverse the effect of the iPLA2b inhibitor BEL on cell cycling, suggesting involvement of additional bioactive mediator of iPLA2b (7).

Regulation of fatty acid availability may represent a critical but previously unrecognized functional aspect of iPLA2b and other LPA-generating enzymes. To test this, we used shRNA knockdown or pharmacological inhibitors of several enzymes involved in hydrolysis of fatty acid and in b-oxidation. Depletion of iPLA2b (even in the presence of LPA), but not MAGL, inhibits growth of ovarian cancer cell lines. Most interestingly, inhibition of carnitine palmitoyl transferase 1 (CPT1), the rate-limiting enzyme of β-oxidation responsible for shuttling long-chain fatty acids into the mitochondrial matrix, with a specific inhibitor etomoxir (8) or shRNA suppressed cell growth (Fig. 1) with limited effect on cell viability in most ovarian cancer cell lines (Fig. 2). However, combination of etomoxir with ABT263, a BH3 mimetic inhibitor of the Bcl2 family members (9) resulted in synergistic induction of apoptosis (Fig. 2). More importantly, we found that CPT1A is overexpressed in most ovarian cancer cell lines compared to normal ovarian epithelial cells (NOE) (Fig. 3, *left*). The sensitivity of ovarian cancer cell lines to apoptosis induced by the combined treatment with etomoxir and ABT263 correlated with the CPT1A expression levels in these cells (Fig. 3, *right*). Taken together, these results indicate that iPLA2b and probably other LPA-synthesizing pathways contribute to ovarian oncogenesis via not only generation of LPA but also enhancement of fatty acid levels for b-oxidation.

2.3 Determination of the effects of LPA signaling on cholesterol synthesis and structures and functions of lipid rafts

We have described in the last report that LPA stimulated expression of cholesterol synthesis rate-limiting enzyme HMG-CoA reductase via activation of SREBP and increased cellular cholesterol levels. We further examined whether change in cholesterol homeostasis could affect the functions of caveolin-1, the principal structural component of the cholesterol/sphingolipid-enriched plasma membrane microdomain caveolae (10). One preliminary observation we made was that LPA induced phosphorylation of caveolin-1 at Tyr14 in ovarian cancer lines. Since caveolin-1 has been implicated as a candidate tumor suppressor (10), we will study whether LPA-mediated phosphorylation affects LPA signal transduction and biological functions in ovarian cancer cell lines.

2.4 Metabolic profiling of alterations in membrane and cellular lipids modulated by LPA using mass spectrometry

During the past year, Abir Mukherjee, one of the former Ph.D. students in the lab received systematic training in lipid quantification with mass spectrometry at the VCU Department of Biochemistry Lipidomics Core. His preliminary analysis of cellular lipid extracts suggest several individual membrane phospholipids, neutral lipids and cholesterol are elevated in LPA-treated ovarian cancer cell lines, consistent with his previous results from spectrophotometric or fluorimeric measurement of total membrane lipids and triacylglycerols in LPA-treated and untreated cells. Unfortunately Mr. Mukherjee recently relocated to University of Chicago for his post-doctoral training before he had opportunity to complete the study. Therefore I have requested a no-cost extension to the end of this year and I have assigned another graduate student F. Yuan to assist me completing this sub-aim.

Key Research Accomplishments (2nd year)

- Identification of LPA as the primary constituent of serum responsible for serum induction of lipogenesis in a number of ovarian cancer cell lines;

- Demonstration of the role of EGF in lipogenesis as compared to LPA. EGF promotes lipogenesis in ovarian cancer cells. A component of the lipogenic activity of EGF could be mediated by autocrine production of LPA;

- Overexpression of autotaxin or incubation with recombinant enzymatically active autotaxin protein is sufficient to stimulate lipogenesis in ovarian cancer cells;

- Identification of a novel functional aspect of iPLA2b and probably other LPA-biosynthesizing pathways, namely upregulation of fatty acids and b-oxidation in addition to LPA production;

- Finding of compelling evidence that b-oxidation of fatty acids plays an active and essential role in ovarian cancer cell proliferation and survival.

Reportable Outcomes

Manuscript published:
Mukherjee A, Wu J, Barbour S and Fang X. Lysophosphatidic acid activates lipogenic pathways and de novo lipid synthesis in ovarian cancer cells. J Biol. Chem. 2012 287:24990-5000. PMID: 22665482

Manuscript in preparation:
Shao H, Mukherjee A, Jing K, Yuan F, and Fang X. Carnitine palmitoyl transferase 1A mediates proliferation and survival of ovarian cancer cells.
Manuscript in preparation.

Conclusions

During the second year of the funding support, we have pursued the specific aims and subaims as planned. The most significant observation we made was that the enzymes accounting for endogenous LPA production also stimulate fatty acid release and catabolic metabolism. Little is known about the role of the resultant fatty acids and b-oxidation in cancer cell growth, survival and carcinogenesis. We obtained strong evidence that in addition to LPA, fatty acids and b-oxidation metabolism play an active role in driving ovarian cancer cell proliferation and resistance to apoptosis. Since we have shown that LPA-driven lipogenesis is required for ovarian cancer proliferation, our results together indicate a dual role for lipid metabolism (anabolism and catabolism) in support of malignant features of ovarian cancer cells.

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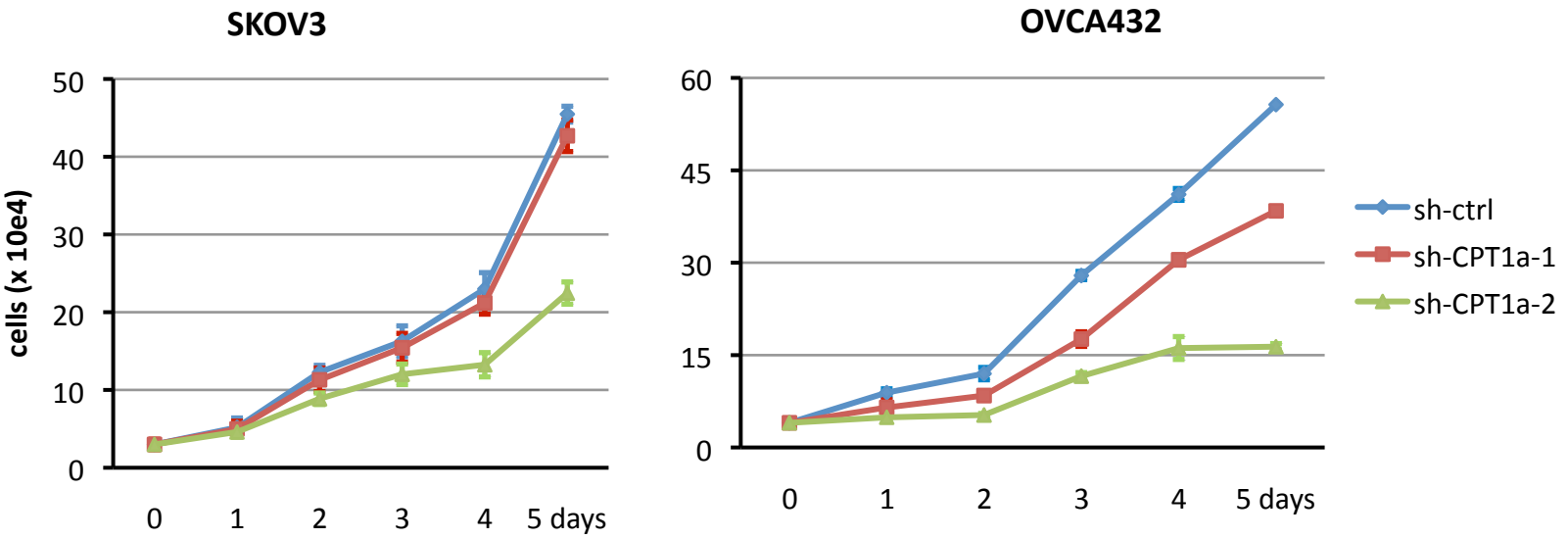
Significant Results



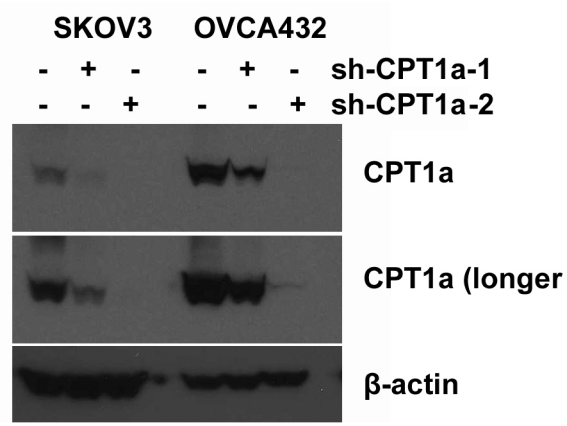


Fig. 1. CPT1A is required for growth of ovarian cancer cell lines. CPT1A in SKOV-3 and OVCA-432 cells was down-regulated by two independent shRNAs. One (sh-CPT1A-2) completely eliminated CPT1A expression while the other (sh-CPT1A-1) partially inhibited CPT1A (lower panel). The growth curves showed a dose-dependent inhibition of SKOV-3 and OVCA-432 cell growth by shRNA knockdown of CPT1A.

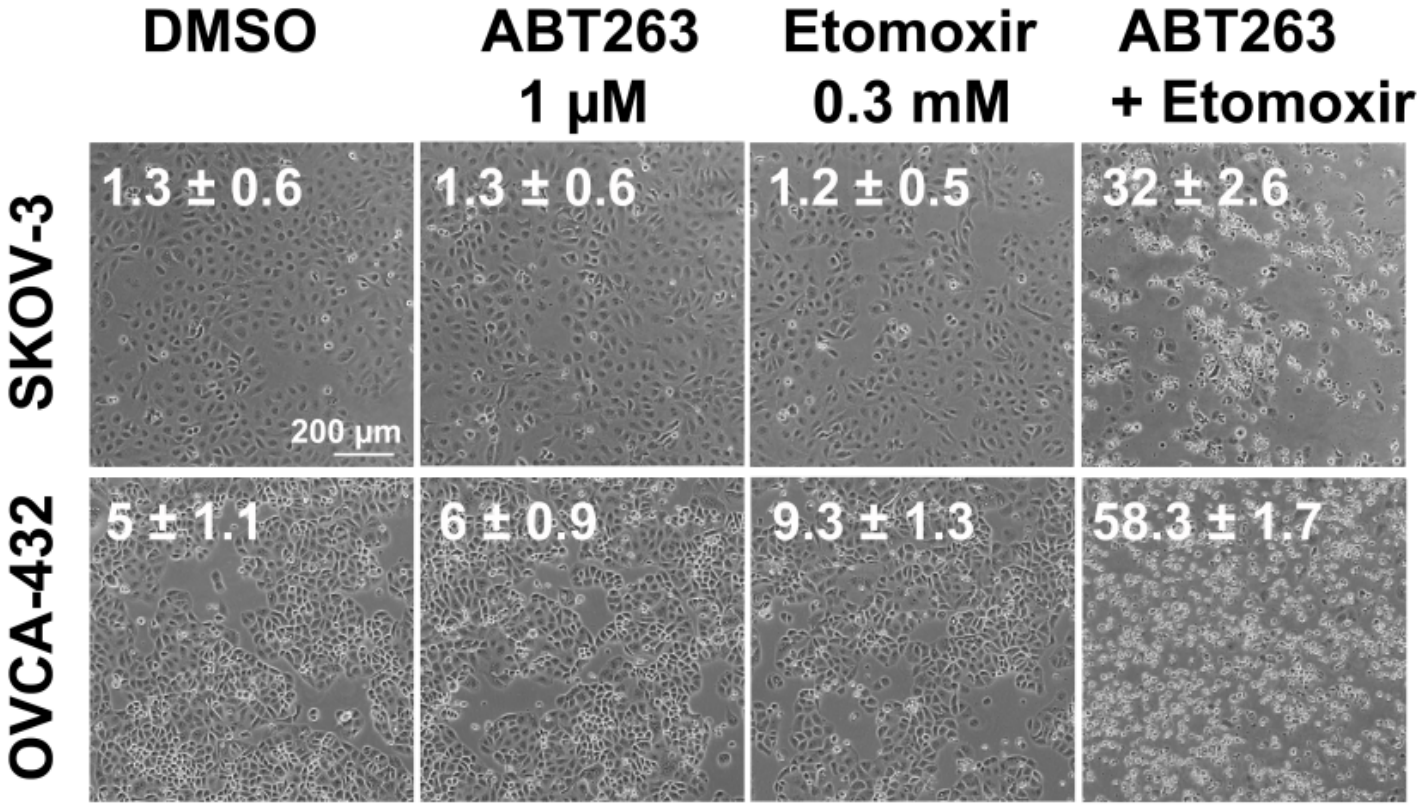


Fig. 2. Co-treatment with etomoxir and ABT263 induced synergistic apoptosis in SKOV-3 and OVCA-432. The cells were treated with ABT263, etomoxir or their combination (Comb) for 24 hours. The percentages of apoptotic cells (presented in each panel) were determined by flow cytometry quantification of Annexin V-positive, apoptotic cells.

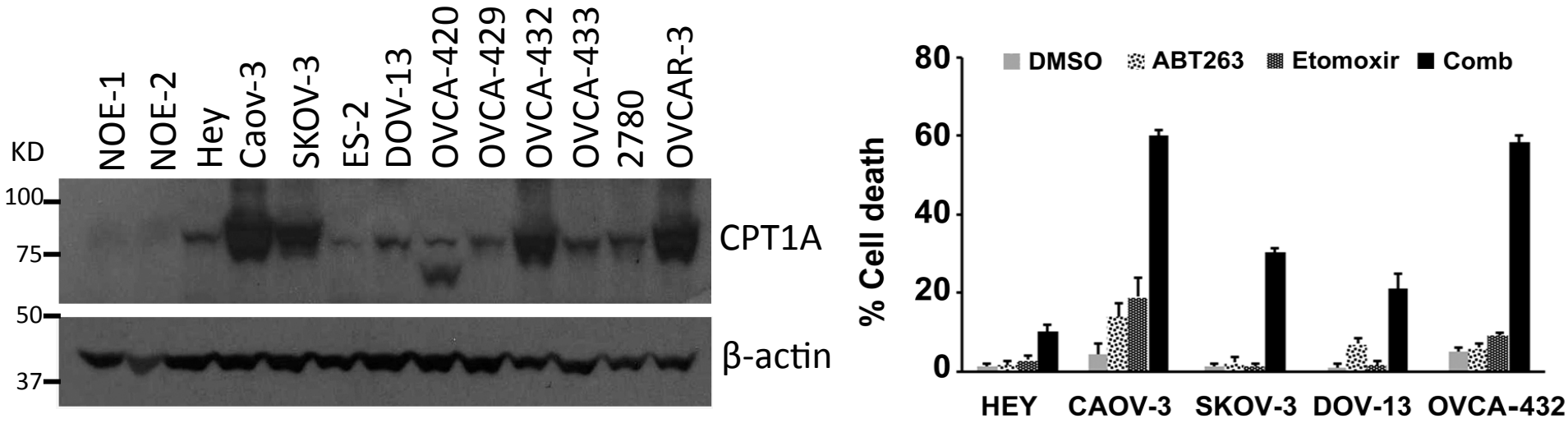


Fig. 3. CPT1A is overexpressed in multiple ovarian cancer cell lines (*left*), which correlates with the sensitivity of the cells to apoptosis induced by combination (Comb) of etomoxir (0.3 mM) and ABT263 (1 μM) (*right*)